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Short communication

Identification of a zoonotic genotype 3 hepatitis E subtype in wildlife in north-eastern Italy

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ABSTRACT

Hepatitis E virus (HEV) is an emerging zoonosis caused by a positive RNA single stranded virus of the Hepeviridae family. In developed countries, pigs and wild boars are the main reservoir for zoonotic genotypes 3 and 4. In spring 2017, for the first time HEV was detected in wild boars captured in the Regional Park of the Euganean Hills, in north-eastern Italy. Phylogenetic analysis of two complete viral genomes and seven partial ORF1 and ORF2 sequences of HEV viruses, selected from twelve positive animals, showed that the viruses grouped together within genotype 3 but clustered separately from previously identified subtypes, thus suggesting the identification of a novel genotype 3 subtype. The phylogenetic analysis of nine partial ORF2 sequences showed the closest similarity with wild boar/human viruses identified in central-northern Italy in 2012. The circulation of HEVs in this area, characterized by a vast man-made environment, an overpopulation of wild boars and > 150 swine farms, should be considered in a public health perspective. Further investigations at the wild/domestic species and human interface are therefore necessary to gain a deeper understanding of HEV dynamics.

1. The study

Hepatitis E is an emerging zoonosis caused by a positive RNA single stranded virus belonging to the *Orthohepevirus A* species of the Hepeviridae family. Of the 8 recognized hepatitis E virus (HEV) genotypes, genotypes 3 and 4 (HEV3 and HEV4) are known for their zoonotic potential and, in developed countries, pigs are their main reservoir. However, cases of foodborne transmission of HEV3 and HEV4 have been reported not only following the consumption of pork liver sausages but also of wild boar products.

HEV actively circulates in the European wild boar populations and the prevalence data are variable according to the geographical area, types of samples processed and analyses performed. In north-western and central Italy wild boar seroprevalence ranges from 5% to 52% and positive rates of viral RNA vary between 0% and 25% (Caruso et al., 2015; De Sabato et al., 2018c; Martelli et al., 2008; Martinelli et al., 2015; Serracca et al., 2015). It is noteworthy that prevalence is influenced by factors that reflect different degrees of interaction between wild boar and domestic pigs, as observed for other pathogens (Linhares et al., 2015).

HEV of genotype 3 is widespread among Italian domestic pigs (Di Bartolo et al., 2008, 2011, 2016; Monini et al., 2015), while only a single detection of genotype 4 was reported in a swine farm in northern Italy in 2015 (Monne et al., 2015). Among genotype 3, subtypes 3c, 3e and 3f have been found in pigs and wild boars in northern Italy (Di Bartolo et al., 2011; Caruso et al., 2015, 2017; La Rosa et al., 2011, 2014, 2017;), subtype 3i in a wild boar in southern Italy (Campania) in 2015 (De Sabato et al., 2018b) and a newly proposed 3l subtype was detected in swine collected in 2012–2013 in northern Italy (De Sabato et al., 2018a). To date little is known about the presence of HEV in the wild population of north-eastern Italy.

In the framework of a HEV surveillance plan carried out in wild ungulates in the Veneto and Friuli Venezia Giulia regions (north-eastern Italy), we identified 12 HEV positive animals out of 138 wild boars sampled between October 2016 and August 2017 in the Regional Park of the Euganean Hills (see Appendix A supplementary data for details). This area covers 18.694 ha, it is divided into 15 municipalities and counts 154 swine farms, 5 of which registered as free-range. Given the various human activities in this area (agriculture, breeding, tourism), interaction between humans, livestock and wild boar is very likely,

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Abbreviations: HEV, Hepatitis E virus; ORF, Open Reading Frame; nt, nucleotide

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Table 1

Sequenced viruses. Sequenced viruses and epidemiological information on wild boars from which the viruses have been isolated.

HEV virus	HEV sequenced genome[a]	Accession number	Positive tissues in real time PCR[b]	Wild boar sex	Wild boar age	Wild boar date of capture	Place of sampling
Wb_Italy_2017_17RS1920_complete	Complete genome	MK390971	liver, bile	female	within 1 year of age	18/05/2017	Cinto Euganeo (Padua)
Wb_Italy_2017_17RS2551-4_complete	Complete genome	MK390970	liver, bile, feces	male	within 1 year of age	09/06/2017	Teolo (Padua)
Wb_Italy _2017_17RS1495–3	Partial ORF1 and ORF2	MK390972 and MK390979	liver, feces	female	within 1 year of age	17/05/2017	Cinto Euganeo (Padua)
Wb_Italy _2017_17RS1546–3	Partial ORF1 and ORF2	MK390973 and MK390980	liver, bile, feces	male	within 1 year of age	08/06/2017	Cinto Euganeo (Padua)
Wb_Italy _2017_17RS1916–6	Partial ORF1 and ORF2	MK390974 and MK390981	feces	female	within 1 year of age	14/06/2017	Teolo (Padua)
Wb_Italy _2017_17RS1917–8	Partial ORF1 and ORF2	MK390975 and MK390982	liver, bile, feces	female	within 6 months of age	15/06/2017	Cinto Euganeo (Padua)
Wb_Italy _2017_17RS1918–6	Partial ORF1 and ORF2	MK390976 and MK390983	liver, bile, feces	male	within 6 months of age	15/06/2017	Cinto Euganeo (Padua)
Wb_Italy _2017_17RS1919–6	Partial ORF1 and ORF2	MK390977 and MK390984	liver, bile, feces	female	within 6 months of age	20/06/2017	Cinto Euganeo (Padua)
Wb_Italy _2017_17RS1921–6	Partial ORF1 and ORF2	MK390978 and MK390985	bile, feces	female	within 6 months of age	15/06/2017	Cinto Euganeo (Padua)

[a] ORF1 (293 nt) position 4273–4565, ORF2 (378 nt) position 5961–6338 with reference to MK390970.

[b] Real Time PCR targeting the ORF3 region as described by Jothikumar et al. (2006).

even more considering that the wild boar population has been increasing since the '90s, when the species was illegally introduced into the Park. As an example, the number of animals culled in January–August 2017 ($n = 1106$) considerably exceeded the number of wild boars estimated in 2007 ($n = 432$). Dealing with this complex epidemiological scenario, the aim of this study was to genetically characterize the wild boar HEVs found in this area and to compare their genetic relatedness with the HEV viruses previously identified in swine and humans.

The complete genomes of two HEV strains and the partial ORF1 (293 nt) and ORF2 (378 nt) sequences of seven HEVs were obtained from nine sub adult (weight < 20 kg) wild boars (Table 1).

We generated three datasets which included subtype reference sequences based on the work by Smith et al. (2016) and sequences resulted from the BLAST search (NCBI, GenBank): 1) partial RNA-dependent RNA polymerase (RdRp) gene in ORF1, 2) partial capsid gene in ORF2 and 3) complete genome alignments. Differently from the previously identified HEV in Italian domestic and wild swine, which belonged to subtypes 3c, 3e, 3f, 3i and 3 l (Di Bartolo et al., 2011; De Sabato et al., 2018a, 2018b; Caruso et al., 2017; La Rosa et al., 2017), the maximum likelihood (ML) phylogenetic tree of the complete genome, obtained in PhyML v.3.1 (Guindon and Gascuel, 2003), showed that the sequences recovered in 2017 clustered together (identity of 99.3%) within genotype 3, but separately from the known subtypes (Figs. 1; 2; S1, Appendix A supplementary data). This phylogeny contains a total of 59 sequences representative of each genetic group of genotype 3 (Fig. 1). Each cluster representative of a different subtype was well supported by bootstrap values ranging from 96.8% to 100%. The cluster of the sequences generated in this study showed a bootstrap support of 100% and a nucleotide p-distance value > 0.143 (between group mean distance) with the twelve genetic subtypes (3a to 3c, 3e to 3 l, and 3ra) and with some unclassified strains. Specifically, it showed the highest similarity with sequences from groups 3i (85.7%) and 3c (85.6%) and with a cluster consisting of two sequences not classified in any subtype (KU176130 and KU513561) (85.6%). Previously identified groups within genotype 3 showed nucleotide p-distance values ranging from 0.125 to 0.213, suggesting that the divergence from other subtype reference strains is sufficient to consider the strains identified in wild boar in the Veneto region as candidates for a novel subtype (Table 2).

The trees of partial RdRp gene (ORF1) and partial capsid gene (ORF2) showed that the 2017 Italian strains clustered together and had a similarity of 99.7%–100% and 98.2%–98.7%, respectively. For the ORF2 region, for which the sequences availability was higher, we observed a close relation with wild boars strains detected in central Italy (Tuscany region) in 2012 (89.4%–92.2% similarity) (Mazzei et al., 2015) and a human case reported in northern Italy (Veneto region) in 2012 (89.7%–90.5% similarity) (Giordani et al., 2013). These events were likely related, as the patient had butchered a wild boar hunted in Tuscany two months before the onset of acute hepatitis.

The ORF2 phylogeny showed that, within most of the genotype 3 subtypes detected in Italy, viruses from wild boars and pigs were clustering together with viruses identified from human cases; this observation was confirmed for the HEVs detected in the Euganean Hills territory. It is well known that HEV spreads to humans via foodborne transmission through faecal contamination of drinking water, consumption of raw or undercooked meat or of raw milk (Huang et al., 2016) from infected animals. Heavy environmental contamination by stool could represent an additive source of infection for forestry workers and hunters (Carpentier et al., 2012). Moreover, an increasing interest for game meat consumption suggests taking this virus into consideration in future risk assessment procedures.

This study represents the first report of the zoonotic HEV-3 subtype in wildlife in north-eastern Italy. We identified a new group, separated from the others by a long branch in all the phylogenetic trees; in the ORF2 phylogeny we observed a wide time gap between the data we

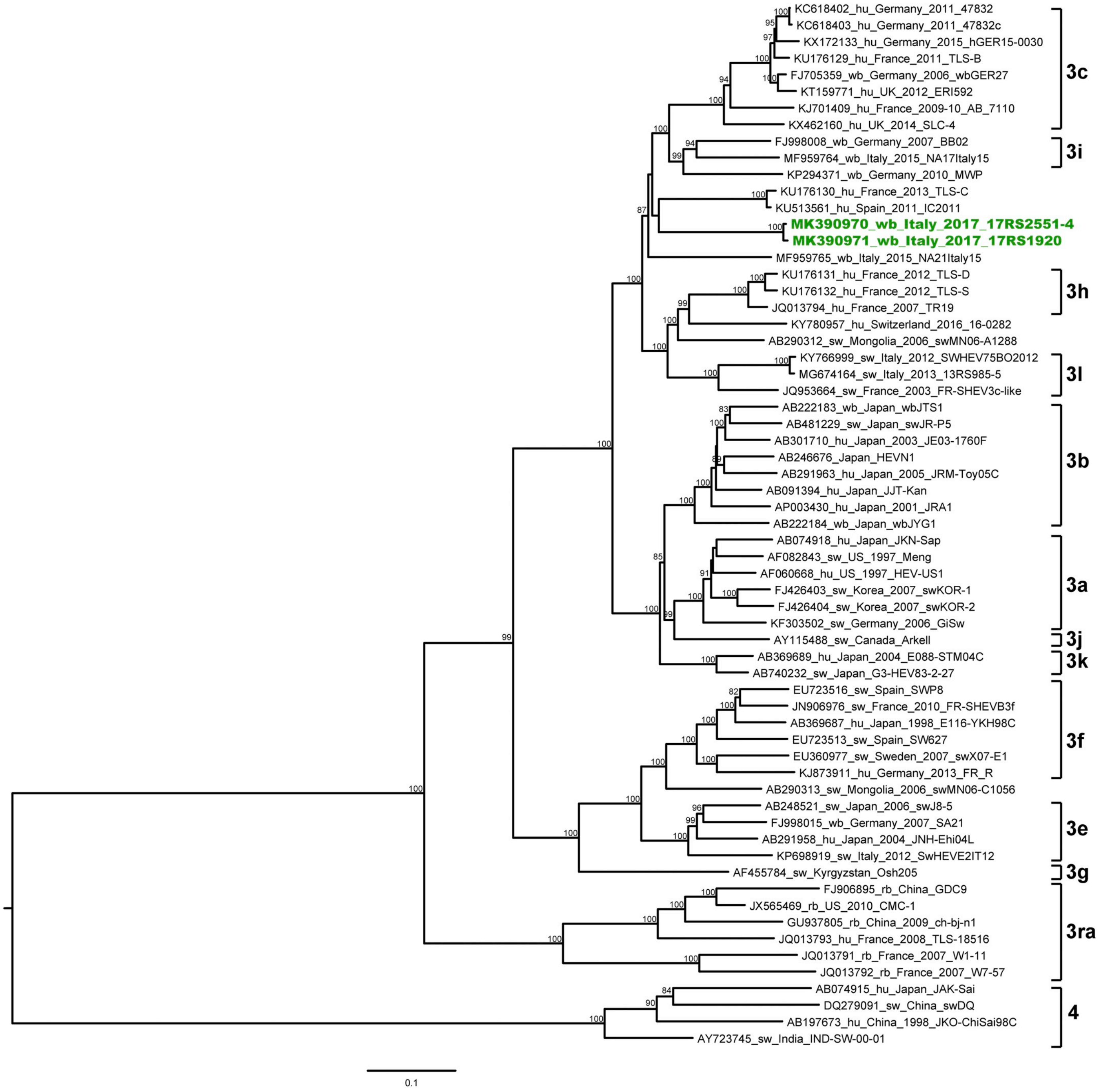

Fig. 1. Maximum-likelihood phylogenetic tree of 59 HEV3 complete genome sequences. The Italian strains obtained in this study are shown in green. Bootstrap supports higher than 80% are reported. Four HEV4 sequences were used as outgroup. Virus names indicate in sequence accession number, host (human (hu), wild boar (wb), swine (sw) or rabbit (rb)), country of origin, year of collection, isolate ID. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

collected in 2017 and the wild boar and human sequences, belonging to the same clade, collected in 2012. These findings suggest that an active surveillance among domestic and wild swine in north-eastern Italy needs to be sustained to determine the source of this new subtype, to monitor the spread of the virus and to prevent transmission to other species, including humans. Furthermore, our results highlight the need to invest in training programs aimed primarily at the categories at risk of exposure to the hepatitis E virus.

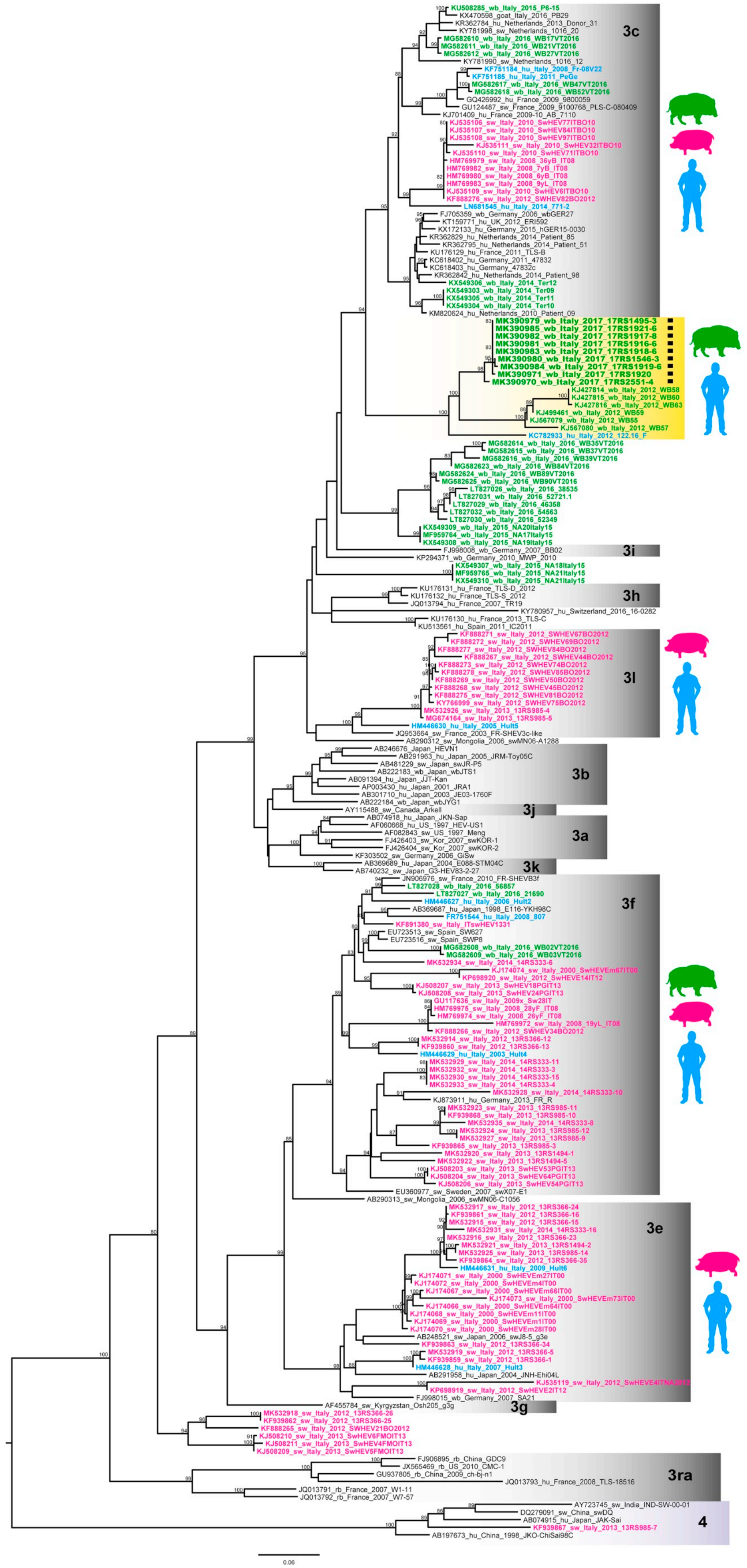

Fig. 2. Maximum-likelihood phylogenetic tree of 196 HEV3 ORF2 (378 nt) sequences. The Italian sequences identified from wild boars, pigs and humans are marked in green, pink and light blue respectively. The different HEV3 subtypes are shown in grey boxes. The yellow box shows the Italian sequences which could represent a potential new subtype. The Italian strains obtained in this study are identified by a black square. Bootstrap supports higher than 80% are reported. Five HEV4 sequences were used as outgroup. Virus names indicate in sequence accession number, host (human (hu), wild boar (wb), swine (sw) or rabbit (rb)), country of origin, year of collection, isolate ID. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

p-distance: between group mean distance. Estimates of evolutionary divergence over sequence pairs between groups calculated in Mega 6. According to the complete genome dataset, sequences belonging to the same g3 genetic group are grouped together, as indicated in Fig. 1.

g3 groups	ITA_2017	g3i	g3c	g3 h	g3 l	g3k	g3a	g3b	g3j	g3e	g3 g	g3f
g3i	0,143											
g3c	0,144	0,133										
g3 h	0,152	0,137	0,145									
g3 l	0,152	0,150	0,150	0,138								
g3k	0,157	0,151	0,155	0,154	0,158							
g3a	0,159	0,152	0,156	0,154	0,158	0,126						
g3b	0,159	0,151	0,155	0,153	0,160	0,128	0,128					
g3j	0,163	0,158	0,160	0,159	0,164	0,132	0,125	0,132				
g3e	0,186	0,185	0,187	0,186	0,185	0,186	0,186	0,185	0,186			
g3g	0,182	0,181	0,179	0,184	0,177	0,182	0,182	0,180	0,182	0,166		
g3f	0,188	0,186	0,187	0,186	0,184	0,187	0,185	0,186	0,189	0,147	0,167	
g3ra	0,212	0,209	0,210	0,211	0,212	0,209	0,211	0,210	0,213	0,208	0,209	0,212

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.005.

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References

Carpentier, A., Chaussade, H., Rigaud, E., Rodriguez, J., Berthault, C., Boué, F., Tognon, M., Touzé, A., Garcia-Bonnet, N., Choutet, P., Coursaget, P., 2012 Sep.. High hepatitis E virus seroprevalence in forestry workers and in wild boars in France. J. Clin. Microbiol. 50 (9), 2888–2893. https://doi.org/10.1128/JCM.00989-12. (Epub 2012 Jun 20. PubMed PMID: 22718947; PubMed Central PMCID: PMC3421804).

Caruso, C., Modesto, P., Bertolini, S., Peletto, S., Acutis, P.L., Dondo, A., Robetto, S., Mignone, W., Orusa, R., Ru, G., Masoero, L., 2015 Jan.. Serological and virological survey of hepatitis E virus in wild boar populations in northwestern Italy: detection of HEV subtypes 3e and 3f. Arch. Virol. 160 (1), 153–160. https://doi.org/10.1007/s00705-014-2246-5. (Epub 2014 Oct 5. PubMed PMID: 25283611).

Caruso, C., Peletto, S., Rosamilia, A., Modesto, P., Chiavacci, L., Sona, B., Balsamelli, F., Ghisetti, V., Acutis, P.L., Pezzoni, G., Brocchi, E., Vitale, N., Masoero, L., 2017 Oct.. Hepatitis E virus: a cross-sectional serological and virological study in pigs and humans at zoonotic risk within a high-density pig farming area. Transbound. Emerg. Dis. 64 (5), 1443–1453. https://doi.org/10.1111/tbed.12533. (Epub 2016 Jul 5. PubMed PMID:27380833).

De Sabato, L., Lemey, P., Vrancken, B., Bonfanti, L., Ceglie, L., Vaccari, G., Di Bartolo, I., 2018 Mr. 15a. Proposal for a new subtype of the zoonotic genotype 3 hepatitis E virus: HEV-3l. Virus Res. 248, 1–4. https://doi.org/10.1016/j.virusres.2018.02.010. (Epub 2018 Feb 13. PubMed PMID: 29452164).

De Sabato, L., Vaccari, G., Lemey, P., Amoroso, M.G., Fusco, G., Ianiro, G., Di Bartolo, I., 2018 Sep 10b. Phylogenetic analysis of two genotype 3 hepatitis E viruses from wild boar, Italy. Virus Genes. https://doi.org/10.1007/s11262-018-1597-5. ([Epub ahead of print] PubMed PMID: 30203361).

De Sabato, L., Ostanello, F., De Grossi, L., Marcario, A., Franzetti, B., Monini, M., Di Bartolo, I., 2018 Sep 11c. Molecular survey of HEV infection in wild boar population in Italy. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12948. ([Epub ahead of print] PubMed PMID: 30207081).

Di Bartolo, I., Martelli, F., Inglese, N., Pourshaban, M., Caprioli, A., Ostanello, F., Ruggeri, F.M., 2008 Nov 25. Widespread diffusion of genotype 3 hepatitis E virus among farming swine in northern Italy. Vet. Microbiol. 132 (1–2), 47–55. https://doi.org/10.1016/j.vetmic.2008.04.028. (Epub 2008 Apr 30. PubMed PMID: 18538512).

Di Bartolo, I., Ponterio, E., Castellini, L., Ostanello, F., Ruggeri, F.M., 2011 May 5. Viral and antibody HEV prevalence in swine at slaughterhouse in Italy. Vet. Microbiol. 149 (3–4), 330–338. https://doi.org/10.1016/j.vetmic.2010.12.007. (Epub 2010 Dec 16. PubMed PMID: 21216541).

Di Bartolo, I., Angeloni, G., Monini, M., Maione, E., Marrone, R., Ostanello, F., Ruggeri, F.M., 2016 Jan 4. Complete genome sequencing of a genotype 3 hepatitis E virus strain identified in a swine farm in Italy. Virus Res. 211, 89–95. https://doi.org/10.1016/j.virusres.2015.09.014. (Epub 2015 Sep 28. PubMed PMID: 26415753).

Giordani, M.T., Fabris, P., Brunetti, E., Goblirsch, S., Romanò, L., 2013 Dec.. Hepatitis E and lymphocytic leukemia in man, Italy. Emerg. Infect. Dis. 19 (12), 2054–2056. https://doi.org/10.3201/eid1912.130521. (PubMed PMID: 24274068; PubMed Central PMCID: PMC3840850).

Guindon, S., Gascuel, O., 2003 Oct.. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52 (5), 696–704 (PubMed PMID: 14530136).

Huang, F., Li, Y., Yu, W., Jing, S., Wang, J., Long, F., He, Z., Yang, C., Bi, Y., Cao, W., Liu, C., Hua, X., Pan, Q., 2016 Aug.. Excretion of infectious hepatitis E virus into milk in cows imposes high risks of zoonosis. Hepatology 64 (2), 350–359. https://doi.org/10.1002/hep.28668. (Epub 2016 Jun 27. PubMed PMID: 27286751).

Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., 2006 Jan.. Hill VR. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J. Virol. Methods 131 (1), 65–71 (Epub 2005 Aug 24. PubMed PMID: 16125257).

La Rosa, G., Muscillo, M., Vennarucci, V.S., Garbuglia, A.R., La Scala, P., Capobianchi, M.R., 2011 Jul. Hepatitis E virus in Italy: molecular analysis of travel-related and autochthonous cases. J. Gen. Virol. 92 (Pt 7), 1617–1626. https://doi.org/10.1099/vir.0.031278-0. (Epub 2011 Apr 6. PubMed PMID: 21471314).

La Rosa, G., Libera, S.D., Iaconelli, M., Ciccaglione, A.R., Bruni, R., Taffon, S., Equestre, M., Alfonsi, V., Rizzo, C., Tosti, M.E., Chironna, M., Romanò, L., Zanetti, A.R., Muscillo, M., 2014 Jul 29. Surveillance of hepatitis A virus in urban sewages and comparison with cases notified in the course of an outbreak, Italy 2013. BMC Infect. Dis. 14, 419. https://doi.org/10.1186/1471-2334-14-419. (PubMed PMID: 25074676; PubMed Central PMCID: PMC4122772).

La Rosa, G., Della Libera, S., Brambilla, M., Bisaglia, C., Pisani, G., Ciccaglione, A.R., Bruni, R., Taffon, S., Equestre, M., Iaconelli, M., 2017 Jun.. Hepatitis E virus (genotype 3) in slurry samples from swine farming activities in Italy. Food Environ. Virol. 9 (2), 219–229. https://doi.org/10.1007/s12560-016-9270-4. (Epub 2016 Nov 16. PubMed PMID: 27853931).

Linhares, M.B., Belloy, L., Origgi, F.C., Lechner, I., Segner, H., Ryser-Degiorgis, M.P., 2015 Mar. 6. Investigating the role of free-ranging wild boar (Sus scrofa) in the re-emergence of enzootic pneumonia in domestic pig herds: a pathological, prevalence and risk-factor study. PLoS ONE 10 (3), e0119060. https://doi.org/10.1371/journal.pone.0119060. (eCollection 2015. PubMed PMID: 25747151; PubMed Central PMCID: PMC4352045).

Martelli, F., Caprioli, A., Zengarini, M., Marata, A., Fiegna, C., Di Bartolo, I., Ruggeri, F.M., Delogu, M., Ostanello, F., 2008 Jan. 1. Detection of hepatitis E virus (HEV) in a demographic managed wild boar (Sus scrofa scrofa) population in Italy. Vet. Microbiol. 126 (1–3), 74–81 (Epub 2007 Jul 10. PubMed PMID: 17706898).

Martinelli, N., Pavoni, E., Filogari, D., Ferrari, N., Chiari, M., Canelli, E., Lombardi, G., 2015 Apr.. Hepatitis E virus in wild boar in the central northern part of Italy. Transbound. Emerg. Dis. 62 (2), 217–222. https://doi.org/10.1111/tbed.12118. (Epub 2013 Jul 19. PubMed PMID: 23865469).

Mazzei, M., Nardini, R., Verin, R., Forzan, M., Poli, A., Tolari, F., 2015 Jun. 4. Serologic and molecular survey for hepatitis E virus in wild boar (Sus scrofa) in Central Italy. New Microb. New Infect. 7, 41–47. https://doi.org/10.1016/j.nmni.2015.05.008. (eCollection 2015 Sep. PubMed PMID: 26199731; PubMed Central PMCID: PMC4506981).

Monini, M., Di Bartolo, I., Ianiro, G., Angeloni, G., Magistrali, C.F., Ostanello, F., Ruggeri, F.M., 2015 Oct.. Detection and molecular characterization of zoonotic viruses in swine fecal samples in Italian pig herds. Arch. Virol. 160 (10), 2547–2556. https://doi.org/10.1007/s00705-015-2538-4. (Epub 2015 Jul 29. PubMed PMID: 26215443).

Monne, I., Ceglie, L., DI Martino, G., Natale, A., Zamprogna, S., Morreale, A., Rampazzo, E., Cattoli, G., Bonfanti, L., 2015 Feb. Hepatitis E virus genotype 4 in a pig farm, Italy, 2013. Epidemiol. Infect. 143 (3), 529–533. https://doi.org/10.1017/S0950268814001150. (Epub 2014 May 15. PubMed PMID: 24831185).

Serracca, L., Battistini, R., Rossini, I., Mignone, W., Peletto, S., Boin, C., Pistone, G., Ercolini, R., Ercolini, C., 2015 Sep. Molecular investigation on the presence of hepatitis E virus (HEV) in wild game in North-Western Italy. Food Environ. Virol. 7 (3), 206–212. https://doi.org/10.1007/s12560-015-9201-9. (Epub 2015 May 26. PubMed PMID:26006251).

Smith, D.B., Simmonds, P., Izopet, J., Oliveira-Filho, E.F., Ulrich, R.G., Johne, R., Koenig, M., Jameel, S., Harrison, T.J., Meng, X.J., Okamoto, H., Van der Poel, W.H., Purdy, M.A., 2016 Mar.. Proposed reference sequences for hepatitis E virus subtypes. J. Gen. Virol. 97 (3), 537–542. https://doi.org/10.1099/jgv.0.000393. (Epub 2016 Jan 7. PubMed PMID: 26743685; PubMed Central PMCID: PMC5588893).